Plant-Microbe Interaction

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Video Article

Plant-Microbe Interaction: Transcriptional Response of *Bacillus Mycoides* to Potato Root Exudates

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Abstract

Beneficial plant-associated bacteria play an important role in promoting growth and preventing disease in plants. The application of plant growth-promoting rhizobacteria (PGPR) as biofertilizers or biocontrol agents has become an effective alternative to the use of conventional fertilizers and can increase crop productivity at low cost. Plant-microbe interactions depend upon host plant-secreted signals and a reaction hereon by their associated bacteria. However, the molecular mechanisms of how beneficial bacteria respond to their associated plant-derived signals are not fully understood. Assessing the transcriptomic response of bacteria to root exudates is a powerful approach to determine the bacterial gene expression and regulation under rhizospheric conditions. Such knowledge is necessary to understand the underlying mechanisms involved in plant-microbe interactions. This paper describes a detailed protocol to study the transcriptomic response of *B. mycoides* EC18, a strain isolated from the potato endosphere, to potato root exudates. With the help of recent high-throughput sequencing technology, this protocol can be performed in several weeks and produce massive datasets. First, we collect the root exudates under sterile conditions, after which they are added to *B. mycoides* cultures. The RNA from these cultures is isolated using a phenol/chloroform method combined with a commercial kit and subjected to quality control by an automated electrophoresis instrument. After sequencing, data analysis is performed with the web-based T-REx pipeline and a group of differentially expressed genes is identified. This method is a useful tool to facilitate new discoveries on the bacterial genes involved in plant-microbe interactions.

Video Link

The video component of this article can be found at https://www.jove.com/video/57606/

Introduction

Plants may exudate up to 20% of the carbon fixed during photosynthesis through roots into the rhizosphere[1], *i.e.*, the narrow zone of soil near the roots. Due to the higher nutrient availability, the rhizosphere is a suitable habitat for diverse microorganisms, including plant-growth promoting bacteria. The root exudates contain a range of inorganic compounds like ions, inorganic acids, oxygen, and water. However, the majority of the root exudates is formed by organic materials, which can be divided into low molecular weight compounds and high molecular weight compounds. The low molecular weight compounds include amino acids, organic acids, sugars, phenolic compounds, fatty acids, and an array of secondary metabolites. The high molecular weight compounds consist of mucilage and proteins[2,3]. Rhizosphere microorganisms can use some of these compounds as an energy source for growth and development. The root exudates play an important role in shaping the rhizobacterial community since the plant-produced compounds in the exudates can influence the behavior of rhizosphere-associated bacteria by affecting the expression of specific genes.

Understanding the bacterial response to root exudates is a key step in deciphering plant-microbe interaction mechanisms. As the bacterial response to plant-microbe interactions is the product of differential gene expression, it can be studied by transcriptome analysis. Using this method, previous studies identified several important genes involved in plant-microbe interactions. In *Pseudomonas aeruginosa*, genes involved in metabolism, chemotaxis, and type II secretion were shown to respond to sugar beet root exudates[4]. Fan *et al.*[5] studied the transcriptomic profiling of *B. amyloliquefaciens* FZB42 in response to maize root exudates. Their results show that, of the genes strongly induced by the root exudates, several groups are involved in metabolic pathways relating to nutrient utilization, chemotaxis, motility, and non-ribosomal synthesis of antimicrobial peptides and polyketides.

The accuracy of these studies relies on the collection of root exudates. Although several methods have described the collection of root exudates for different purposes, they either demand sophisticated instruments or are not performed in well-controlled conditions[6,7,8]. Moreover, rhizosphere-inhibiting microorganisms can influence root exudate composition by affecting plant cell membrane permeability and damaging the root tissues, particularly in the case of consortia of microorganisms[9]. When investigating the microbial response to root exudates, it is important to use well-defined conditions in order to avoid alteration of the compounds by other microorganisms[10]. Furthermore, high-quality

RNA is required for RNA-seq based transcriptome studies. However, when dealing with non-model-bacterial strains, the standard protocols or commercial kits usually have a low efficiency due to unknown factors or special growth properties.

The protocol described here was verified using *B. mycoides*, which is a gram-positive, spore-forming bacterium of the Firmicute phylum. It is ubiquitous in the rhizosphere of various plant species. Several plant growth promoting properties have been reported for this species, including induction of systematic resistance (ISR) in sugar beet[11], inhibition of the damping-off pathogen *Pythium* for cucumber[12], as well as nitrogen fixation in the sunflower rhizosphere[13]. However, the molecular mechanisms of its interaction with a host plant are not well studied.

The objective of the experiments presented here is to study the transcriptomic response of endosphere-isolated *B. mycoides* to potato root exudates. In short, the protocol consists of the following steps: first, collect potato root exudates under sterile conditions. Then, extract high-quality RNA from bacterial cells treated with root exudates. The final step is data analysis using the web-based T-REx pipeline[14]. This protocol was used to identify *B. mycoides* genes that show a shift in expression levels upon contact with root-exudates and thus might play an important role in plant-microbe interactions.

Protocol

1. Germinating Potato in Sterilized Conditions

1. Rinse the potato surface with sterile water. Bathe the potato in 70% ethanol and then in 3% sodium hypochlorite, for 5 min each. Rinse it again with sterile water to remove any remaining sodium hypochlorite.
2. Prepare the materials needed for germinating and growing potato tubers; sterilize the plastic pots, engraftment baskets, vermiculite, and water by autoclaving them at 121 °C for 20 min.
   NOTE: Ensure that all materials used are autoclavable. Otherwise, use other sterilizing methods.
3. Put the surface-sterilized potato into the engraftment basket, and place this in an autoclaved pot containing wet vermiculite.
4. Keep the pot in a climate chamber at 24 °C for three weeks. Set it on cycles of 16 h of light (120 µmol $m^{-2}s^{-1}$) and 8 h of darkness. To avoid microbial contaminations, use a large glass fiber box to cover the pot.

2. Collecting Potato Root Exudates

1. When the shoots sprout, transfer the basket with the whole potato into a sterilized beaker filled with 150 mL of autoclaved deionized water, with the potato tubers placed just above the water surface and the roots submerged in the water (see **Figure 1**). Keep the seedling in the climate chamber, and use the same settings as before [24 °C; cycles of 16 h of light (120 µmol $m^{-2}s^{-1}$), 8 h of darkness].
2. From the second day on, collect the water containing the exudates, and refill the beaker with sterile deionized water. Perform the sampling every day until the seventh day after transferring the seedling.
3. Store each sample separately at 4 °C. For each sample, spread 100 µL on a Luria-Bertani (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl) agar plate to check for microbial contaminations. Discard the contaminated samples.
4. Combine the collected samples of one seedling, and concentrate them by freeze-drying them at -40 °C to a final volume of 150 mL.

3. Growing Bacteria

1. Streak a *B. mycoides* strain from a -80 °C glycerol stock onto an LB agar plate, and incubate the plate at 30 °C overnight.
2. Inoculate an LB liquid medium with a single colony from the plate, and grow the culture in a shaking incubator at 200 rpm overnight at 30 °C.

4. Treatment and Sampling of Bacteria

1. To compare the growth curve of the bacteria treated with root exudates to a negative control, prepare a series of flasks containing 50 mL of LB liquid medium. Add 0.5 mL of the overnight bacterial culture to all flasks, and add either 0%, 5%, 10%, or 15% (v/v) root exudate or sterile deionized water to the medium, respectively.
2. Use a culture with deionized water instead of root exudates as a control. Measure the optical density at 600 nm (OD_{600}) every 1 h afterward. Generate a growth curve by plotting the OD_{600} values versus time.
   NOTE: We saw that 10% of the root exudates did not affect the growth of *B. mycoides* (see **Figure 2**); this ratio was used for RNA-seq experiments.
3. Dilute an overnight *B. mycoides* culture with 90 mL of pre-warmed LB medium to the initial OD_{600} of ~ 0.05 in a 300 mL flask. Add 10 mL of root exudates to the culture and incubate it at 30 °C for 1 h.
4. Use a 10 mL sterile deionized water treatment as a control. Collect cells from the culture by centrifugation at 9,000 x g for 2 min at 4 °C. Discard the supernatant, and immediately freeze the pellet in liquid nitrogen, then store it at -80 °C until use.

5. RNA Isolation

NOTE: Before starting the isolation, prepare the workbench, racks, and pipettes by cleaning them with an RNase decontamination solution (see **Table of Materials**). Wear gloves at all time, and make sure all tubes, tips, and solutions are RNase-free. Always keep the samples on ice when possible.

1. Add 0.1% (v/v) of diethyl pyrocarbonate (DEPC) to ultrapure water (100 µL of DEPC per 100 mL solution), mix it well, and keep it at room temperature overnight. Autoclave the mixture to inactivate the DEPC after overnight treatment. Use this DEPC-treated ultrapure water to prepare the TE buffer [10 mM Tris-HCl (pH = 8); 1 mM EDTA] and a 10% SDS (w/v) solution.

2. Thaw the cell pellets on the ice and suspend each in 400 µL of TE (DEPC) buffer. Transfer the suspensions into 2 mL screw cap tubes.
3. Premix 300 µL of chloroform-isoamyl alcohol (24:1 v/v) and 300 µL of phenol (acid phenol, RNA grade), and allow the mixture to stand for 5 min. Take 500 µL of the organic upper phase to add to the resuspended cells. Then add 50 µL of 10% SDS and 0.5 g of glass beads (0.5 µm) into the tube.
4. Close the cap firmly and place the tube in a bead-mill homogenizer (see **Table of Materials**). Perform a 3× 45 s pulse homogenization with a 1 min interval on ice.
5. Centrifuge the samples for 10 min at 11,000 x g (4 °C), and transfer the upper phase to a new tube.
6. Add 500 µL of chloroform-isoamyl alcohol (24:1) to the upper phase from step 5.5 and centrifuge the mixture for 5 min at 11,000 x g (4 °C).
   NOTE: The following steps were modified from the manufacturer's instruction of a commercial glass fiber filter-based RNA isolation kit (see **Table of Materials**).
7. Transfer 500 µL of the upper phase to a fresh tube, add 2 volumes (1 mL) of a lysis/binding buffer and mix it by pipetting up and down.
8. Combine the filter and collection tubes (from the RNA isolation kit) and pipette the mixture from step 5.7 to the filter tube. Centrifuge the tubes for 15 s at 8,000 x g and discard the flow-through.
9. Prepare 1.5 mL microcentrifuge tubes with 100 µL of DNase buffer, 10 µL of DNase I, and 5 µL of an RNase inhibitor (see **Table of Materials**). Add the prepared solution mix on the filter of the filter tube, and incubate it for 20 - 30 min at 15-25 °C.
10. Perform the washing steps according to the manufacturer's instruction. Use 50 µL of an elution buffer to elute the RNA.
11. Save the eluted RNA at -80 °C, and put a plastic paraffin film around it. At the same time, transfer a few microliters of the sample to a 1.5 mL microcentrifuge tube to perform a quality check.

6. RNA Quality Check and Sequencing

1. Check the RNA with a microvolume spectrophotometer.
   NOTE: Absorbance ratios at 260/280 should be above 1.8, and ratios at 260/230 should be above at least 1.8, preferably above 2.0.
2. Run the purified RNA in an automated electrophoresis instrument using the recommend RNA analysis kit (see **Table of Materials**); the RNA integrity number (RIN) must be at least above 7 to proceed.
3. Use a total amount of 3 µg of RNA per sample to generate libraries with an RNA-Seq library preparation kit (see **Table of Materials**) following the manufacturer's recommendations.
   NOTE: The library preparations were sequenced on a high-throughput sequencing platform and paired-end reads were generated.

7. Data Analysis Using the Web-Based Pipeline T-REx

1. Perform a quality filtering using the FASTX-Toolkit version 0.0.13 (Phred quality scores of > 20). Trim the raw RNA-Seq reads from the adapter sequences with the Trimmomatic tool.
   NOTE: All the downstream analyses were based on clean data with high quality.
2. Download the *B. mycoides* ATCC 6462 NCBI genome database (NCBI accession No.: CP009692.1) and use it as a reference genome for mapping the clean reads using Bowtie2/2.2.3.
3. Use HTSeq v0.6.1 to count the reads numbers mapped to each gene and the reads per kilobase of transcript per million mapped reads (RPKM) of each gene based on the length of the gene and reads count mapped to this gene.
4. For the analysis of the data by the T-REx pipeline, use the RPKM table as input, as well as three descriptive files: (i) a factors file to describe the experiment in factors, (ii) a contrasts file to define which factors will be compared for differential gene expression, and (iii) a class file to describe groups of genes of interest.
   NOTE: The descriptive files are in.txt format. Examples can be found on the webpage of T-REx (http://genome2d.molgenrug.nl/). A detailed instruction for data analysis using T-REx can be found in a previous publication by de Jong *et al.*[14].

Representative Results

Plant-associated microorganisms can positively influence plant growth and health. However, the mechanisms of the complex interactions between plants and their microbial symbionts are not fully understood. Root exudates play an important role in regulating the rhizobacterial activity and behavior, and it is generally postulated that the microbial colonization of roots initiates with the attraction of microbes to root exudates. The aim of this work was to investigate the transcriptomic response of rhizobacterial *B. mycoides* to potato root exudates. To fulfill this, potato tubers were surface sterilized and germinated in autoclaved vermiculite. Then the root exudates were collected as shown in **Figure 1**. In order to rule out the possibility of the root exudates affecting the bacterial growth, up to 15% of the root exudates were added to the *B. mycoides* culture, and no change in growth was detected during the measuring time (**Figure 2**). Thus, the gene expression changes observed in this study were not likely caused by growth-related effects.

After collection, the root exudates were added to the *B. mycoides* culture at a 10% ratio (v/v), and the bacterial total RNA was isolated as previously described. The RNA was then subjected to a quality check by an automated electrophoresis instrument of which the results are shown in **Figure 3**. **Figure 3A** and **3B** represent the RNA isolated from *B. mycoides* treated with the root exudates, and **Figure 3C** and **3D** represent the RNA isolated from the control group. All samples scored a RIN value above 9 with two clear bands corresponding to the 16S and 23S RNA subunits, demonstrating that high-quality RNA was obtained by this protocol.

After library preparation, pair-end reads were obtained with a high-throughput sequencing platform. The raw RNA-Seq reads were trimmed from the adapter sequences and mapped against the reference genome sequence. Of the resulting data, the RPKM table was generated. The transcriptome analysis was performed with the T-REx pipeline. The ratio intensity plot of all the differentially expressed genes is shown in **Figure 4**. As compared with a control, the addition of potato root exudates induced 715 genes to be differentially expressed. Of those, 408 genes were upregulated, and 307 genes were downregulated[15]. The relative change of some of the genes with altered expression is listed in **Table 1**.

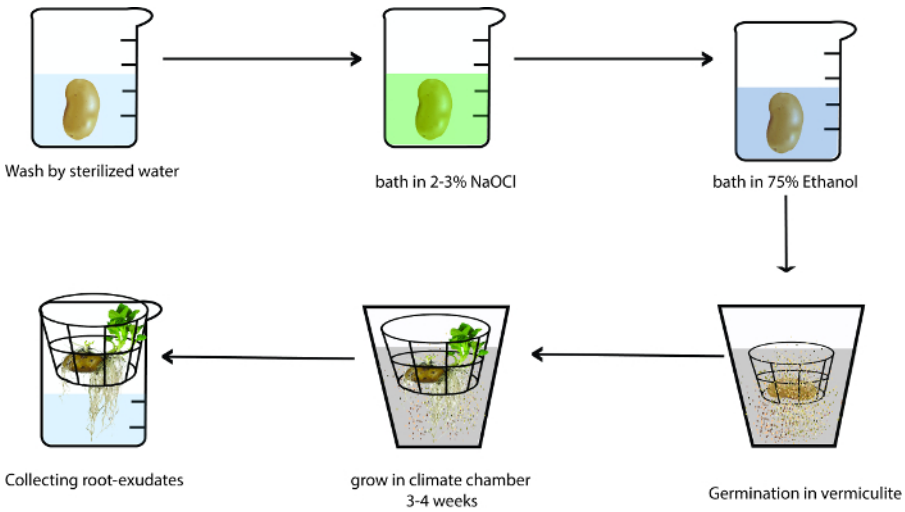

Figure 1: Process scheme of the collection of potato root exudates. The materials used are autoclaved and the germination is performed in a climate chamber. Wash the potato tuber with sterilized water, and bath it in 2-3% NaOCl for 5 min. Bath it in 75% ethanol for another 5 min. Place the potato into an autoclaved basket and put it into a pot containing wet vermiculite. Grow the potato in a climate chamber for 3 - 4 weeks, and then transfer the basket with the potato seedling to a beaker. Collect the root exudates every day and refill the beaker with sterilized water. Please click here to view a larger version of this figure.

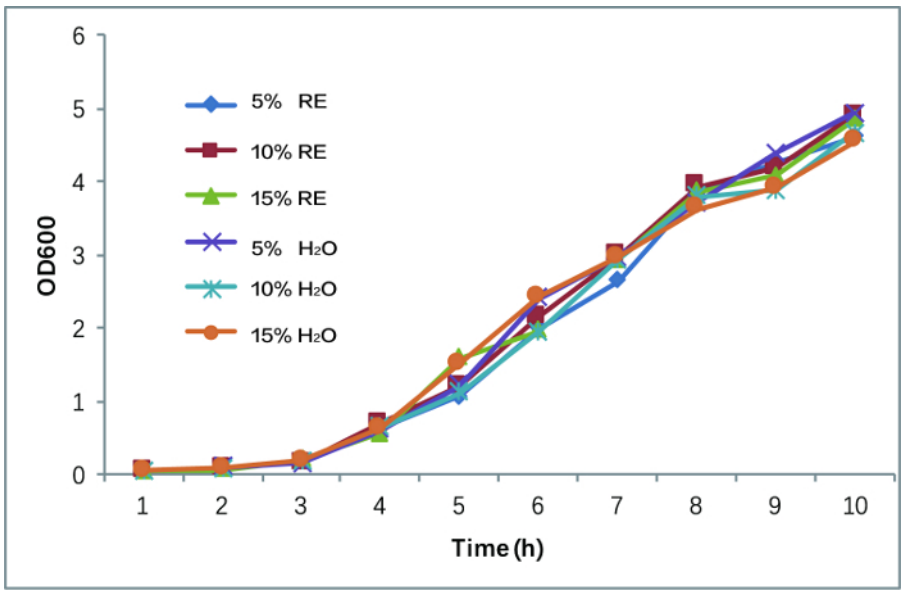

Figure 2: The growth curve of *B. mycoides* with different concentrations of potato root exudates. The strain EC18 was grown in a liquid LB medium with the addition of potato root exudates or sterile H_2O. OD_{600} was measured every 1 h and plotted versus time. All the groups show a similar growth pattern, indicating that up to 15% of the root exudates addition has no significant effects on *B. mycoides* growth. Please click here to view a larger version of this figure.

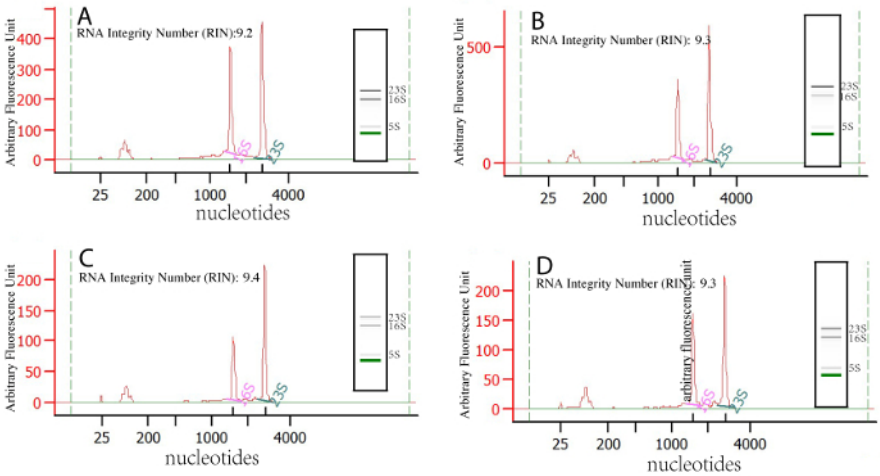

Figure 3: RNA quality check by the automated electrophoresis instrument. A and **B** represent the RNA isolated from the *B. mycoides* treated with root exudates and **C** and **D** represent the RNA isolated from the control group. All the RNA samples show two clear bands corresponding to 23S and 16S rRNA and a weak 5S rRNA band. Please click here to view a larger version of this figure.

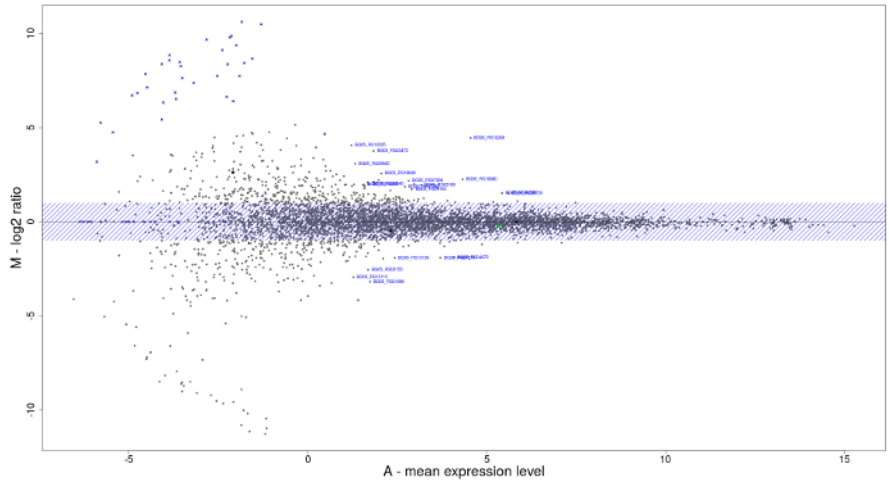

Figure 4: Ratio intensity plot for visualizing differential gene expression of RNA-seq samples of *B. mycoides* in response to potato root exudates. The figure is automatically generated by T-REx. The X-axis represents the gene expression level, and the Y-axis represents the log2-transformed fold change. The genes being up- and downregulated have positive and negative log2 ratio values. The dots in the striped area indicate genes that are not significantly over- or under-expressed. Please click here to view a larger version of this figure.

Gene tag	Strand	Fold change	Annotation
BG05_RS09165	+	3.3	multidrug efflux protein
BG05_RS20930	+	25.3	membrane protein
BG05_RS10935	+	23.3	stage III sporulation protein AD
BG05_RS08990	-	11.8	sporulation protein
BG05_RS16405	+	3.9	IclR family transcriptional regulator
BG05_RS24905	-	3	tryptophan synthase subunit alpha
BG05_RS24920	-	2.3	indole-3-glycerol phosphate synthase
BG05_RS22255	-	27	acetolactate synthase
BG05_RS22250	-	6.5	ketol-acid reductoisomerase
BG05_RS22265	-	26.4	branched-chain amino acid aminotransferase
BG05_RS18715	-	2.8	pullulanase
BG05_RS18040	+	-9.1	germination protein YpeB
BG05_RS16930	+	-4.2	sugar ABC transporter ATP-binding protein
BG05_RS27345	+	-2.9	MFS transporter
BG05_RS19555	-	-3.3	PTS cellobiose transporter subunit IIB
BG05_RS24345	-	-2.7	putrescine importer
BG05_RS22525	-	-12.4	cardiolipin synthase
BG05_RS15225	-	-3.3	membrane protein
BG05_RS18475	+	-5.7	membrane protein
BG05_RS19095	+	-2.1	germination protein

Table 1: List of differentially expressed genes of a root exudates-treated group in comparison with a control.

Discussion

Plant-microbe interactions have been hypothesized to be determined by a finely tuned equilibrium between bacteria and plants. Such interactions are highly complex and difficult to study in a natural system, which comprises diverse microbial species, potentially acting as consortia. This paper describes a simplified protocol to study the bacterial response to root exudates under well-controlled conditions. The transcriptome profile of rhizobacteria, upon exposure to root exudates, provides detailed information on bacterial adaptation to the rhizosphere niche. This root exudates collection protocol does not require complicated procedures and specialized equipment. However, all procedures must be carried out under strictly sterile conditions and a sterility control should be included. We recommend growing several potato tubers in parallel and discarding the contaminated ones. Modifications can be made to this protocol if other plant species are being studied. It is important to use an appropriate method to sterilize any seeds/tubers because they may vary in tolerance to the disinfectants applied.

Once the root exudates are obtained, high-quality RNA must be isolated from the bacterial cells. Various reagents and standard protocols that are primarily based on the phenol/chloroform or TRIzol method are time-consuming[16]. The commercial kits are mostly designed for model organisms but are less applicable to others. This RNA isolation protocol that combines the phenol/chloroform method and an RNA isolation kit overcomes the disadvantages of these methods. Moreover, a bead-beating step is included to homogenize *B. mycoides* cells that typically aggregate in the planktonic culture. Potential DNA contamination is removed by an extra incubation with DNase prior to elution. The high RIN number implies the isolation of intact RNA. Thus, this protocol is especially efficient and time-saving for environmental bacterial strains.

After RNA sequencing, data analysis is performed with T-REx, a web-based statistical analysis pipeline for RNA-seq gene expression data[14]. This pipeline is user-friendly, especially for biologists without extensive bioinformatics knowledge. The input file is the raw RNA expression level data, such as RPKM, fragments per kilobase per million mapped reads (FPKM), counts per million mapped reads (CPM), or other gene expression units. Such gene expression value files can be generated by available tools including SAMtools[17], BEDtools[18], and NGS-Trex[19]. In order to run the RNA-seq analysis, three other input files are needed. These files are used to define the factors that describe the experiments and the replicates, the comparisons between the various experimental conditions, and the groups of genes of interest. When the input files are uploaded, the analysis process will only take a couple of minutes.

Several differentially expressed genes of *B. mycoides* EC18, when treated with root exudates, are listed in **Table 1**. Among them, the expression of several genes encoding membrane proteins is altered. Genes related to the sporulation or germination process are differentially expressed. The expression of genes involved in sporulation also changes in *B. subtilis* when co-cultured with rice seedlings, because root exudates supply the energy required for the dynamic growth of bacterial cells[18]. The expression of the IclR transcriptional regulator, which is related to multidrug resistance and the degradation of aromatic compounds in soil bacteria, is upregulated. The IclR deletion strain of rhizobacteria *Klebsiella*

pneumoniae has decreased the mineral phosphate solubilization, compared with the wild-type strain[19]. Several genes related to amino acids metabolism and synthesis are stimulated, while genes involved in sugar transport including a cellobiose PTS transporter are downregulated. This suggests that strain EC18 may have a metabolic preference for amino acids over sugars in the rhizosphere. The function of the altered genes can be further studied *in situ* by making knockout or overexpression mutants. In summary, the protocol described here enables a quick identification of a large number of potentially important bacterial genes involved in plant-microbe interactions.

Disclosures

The authors declare that they have no competing financial interests.

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